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Comparison of the Microbial Community Composition at Yucca Mountain and Laboratory Test Nuclear Repository Environments

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Comparison of the Microbial Community Composition at Yucca Mountain and Laboratory Test Nuclear Repository Environments

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ABSTRACT

The microbiological community structure within a proposed nuclear waste repository at Yucca Mountain (YM), NV was determined. Microbial growth from collected rock was detected using simulated ground water as a growth medium, with or without amendment of a carbon source. Grown isolates were identified by 16S ribosomal DNA (rDNA) sequence analysis. A more complete compositional analysis of the microbial community located at the proposed nuclear waste repository site was performed using environmental DNA isolation and subsequent identification of amplified 16S rDNA genes. Concurrently, a series of corrosion testing tanks that simulate the evolution of anticipated environmental conditions within the proposed repository have been subjected to the same type of analyses.

KEYWORDS

MIC; Yucca Mountain; bacterial community profile; biodiversity; nuclear waste

INTRODUCTION

The U.S. Department of Energy is evaluating packaging materials to contain high-level nuclear waste at a proposed repository site located at Yucca Mountain, NV (YM). Microorganisms, both those endogenous to YM and those introduced through construction activities or infiltration, could possibly cause Microbiologically Influenced Corrosion (MIC). The abilities of the microorganisms that are present to affect MIC will be dependent whether their metabolic activities promote corrosion and on conditions in the surrounding environment which would permit potentially corrosive activities to occur.

Initially, studies described herein indicated that water availability is presently the primary growth limiting factor at YM. Repository conditions are expected, however, to change dramatically over time after nuclear waste emplacement, including high relative humidities in regions of elevated water infiltration[1]. Organisms grown from YM rock using simulated YM groundwater as a growth medium showed only a limited number of types of bacteria. However, only a small fraction (approximately 1%) of the total number of types of organisms present in an environmental sample can be isolated using a given growth medium[2]. Methods that preclude the need to culture organisms were therefore undertaken to identify YM microorganisms. A complete roster of the organisms extant within YM was thus generated to provide more information regarding metabolic activities that might potentially cause MIC.

After nuclear waste packages are initially emplaced in the prospective repository, gamma radiation fields would be expected to sterilize the region immediately surrounding the packages. As radionuclide decay progresses and accompanying temperatures decrease, water is anticipated to re-infiltrate through rock fractures. Both infiltrating water and incoming air currents can introduce microorganisms back into repository drifts. Thus, after radiation has subsided, those organisms capable of surviving through the extremes of radiation, heat and desiccation, as well as those that infiltrate with water and air are anticipated to inhabit the repository. Those organisms that are capable of growth will be those that best adapted to exploiting resources under repository environmental conditions.

A long-term corrosion experiment, currently being conducted at Lawrence Livermore Natl. Laboratory (Livermore, CA) includes environments that are expected during repository evolution. Large tanks (1,000 L capacity) contain candidate waste package material coupons in solutions that vary in ionic strength and composition (simulated dilute groundwater, concentrated groundwater), pH, and temperature (60° or 90° C.). While deionized water was used in the composition of tank solutions, microorganisms were not purposefully excluded, and the tanks are not fully sealed from the surrounding laboratory environment. Therefore, these test tanks pose a somewhat analogous environment to the evolving repository. Initially the tanks and their contents were sterile or harbor very few organisms, but due to their exposure to the surrounding environment, they have the potential of being colonized by organisms able to survive and grow under the specific conditions prevailing in the tanks. After preliminary experiments employing differential staining and epifluorescence microscopy, it was established that microorganisms were present in tanks with acidic water, as well as dilute and concentrated waters at 60° C. Here, further exploration of the types of organisms present in Long Term Corrosion Tanks (LTCT) was conducted using direct DNA extraction and characterization.

EXPERIMENTAL PROCEDURES

Sample Collection

Samples of Topopah Springs tuff, a welded volcanic ash, were aseptically collected from subsurface regions encompassing the geologic horizon (Alcove 5) of a proposed nuclear waste repository in the Exploratory Studies Facility (ESF), Yucca Mountain (YM), NV. The ESF is an eight km tunnel that provides access to the repository horizon for study and analysis of the area. The potential repository is proposed to be located approximately 300 meters below the surface of YM (in the Lower Lithophysal geologic unit) and connected to the existing ESF. Sterilized rock hammers, forceps, and coring tools were employed in sampling YM tuff to prevent introduction of potential contaminants during collection. When required, collected rock was aseptically crushed and sieved (Enterpac model RR308, 2-4mm grain size); crushed and sieved rock from all collected samples were blended together by homogenizing (15-20 revolutions/minute) before use. Before and after crushing, rock samples were maintained in sterilized sealed containers at room temperature. Crushed rock used for sterile control experiments was gamma-irradiated using a ^{60}Co source (total dose of >3Mrad).

Conditions to Assess Microbial Growth in Groundwater

Simulated groundwater ("10XJ13 Synthetic" medium) was formulated from a ten-fold concentration of YM ground water using *The Geochemist's Workbench*[3,4] (Table 1). 10XJ13 Synthetic medium contained, per liter, 0.049 g NaF, 1.9 mL 1.0M HCl, 0.25 mL 0.1M $CaCl_2$, 0.044 g $NaHCO_3$, 1.73 mL 0.1M H_2SO_4, 0.14 g Na_2SO_4, 0.095 g $MgSO_4$, 0.021 g $NaNO_3$, 0.132 g KNO_3, 2.24 mL 1M NaOH. 10XJ13 Synthetic medium was supplemented with 0.1% glucose except as specified; the final pH was 7.8.

Growth rates of YM bacteria contained in YM tuff were determined by periodically measuring cell densities from batch flask and microcosm-grown cultures. Aseptically crushed and homogenized YM tuff (containing endogenous microorganisms) was added to 125 mL flasks in a 1:4 ratio of tuff: indicated media, and incubated at 30°C., with continuous agitation (150 rpm).

Cell densities were determined by live plating techniques. The aqueous phase (i.e., planktonic cells) of either batch or microcosm-grown cultures were periodically sampled, serially diluted, and plated on R2A agar (Difco). Plates were incubated at 30° C. for 24-36 h., or until colonies were large enough to be enumerated.

Identification of Isolated Organisms

Individual morphotypes were single colony-purified from R2A agar plates used for cell density determinations (above). Isolates were initially gram stained and then subjected to Biolog analysis according to the manufacturer's protocol[5] (Biolog, Inc., Hayward, CA) for the purpose of eliminating duplicate species among isolated organisms. Isolates that showed unique patterns of carbon utilization by Biolog analysis were subjected to 16S ribosomal DNA (rDNA) sequence determination and analysis to identify unique isolated organisms[6] (Midi Labs, Inc, Newark, DE).

Table 1. Comparison of Natural J13 Groundwater[a] (Concentrated 10-Fold) with Simulated J13 Water (Concentrated 10-Fold)

Component	1×J13[a] groundwater	1×J13 synthetic water
	Concentration (mM)	Concentration (mM)
Na	1.910	0.614
K	0.131	0.131
Ca	0.312	0.251
Mg	$7.90X10^{-2}$	$7.90X10^{-2}$
NO_3	0.155	0.155
Cl	0.195	0.195
CO_3	2.050	$5.30X10^{-2}$
SO_4	0.195	0.195
Li	$6.05\ X10^{-3}$	0
Sr	$3.99\ X10^{-4}$	0
Al	$4.45\ X10^{-4}$	0
Fe	$1.07\ X10^{-4}$	0
Si	0.961	0
F	0.116	0.116
Glucose	0	0.555

[a] J.M. Delaney, "Reaction of Tonopah Spring Tuff with J-13 Water: A Geochemical Modeling Approach Using EQ3/6 Reaction Path Code," UCID-53631 Lawrence Livermore National Laboratory (1985).

DNA Extraction from YM rock

Genomic DNA was extracted using a modification of the method outlined by Zhou *et al.*[7] DNA was extracted from 400 g (dry weight) of aseptically weighed, crushed YM rock. YM rock was divided into 2 x 200 g samples. To each 200 g sample, 81 ml of DNA extraction buffer [100 mM Tris (pH 8), 100 mM EDTA (pH 8), 100 mM $NaPO_4$ (pH 8), 1.5 M NaCl, and 1% hexadecylmethyl ammonium bromide (CTAB)], 600 µl of a proteinase K solution (10 mg/ml), and 6.75 ml Inhibitor Removal Solution solution (IRS, MO BIO Laboratories, Inc., Solano Beach, CA), was added. After extraction, the slurry was

pelleted by centrifugation at 6,000 *X g* for 5 min. at room temperature, and the supernatant was decanted by passing over sterile gauze to remove rock fines. A second genomic DNA extraction was performed on the rock samples by adding 25 ml of DNA extraction buffer, 200 µl proteinase K, 2.25 ml of IRS solution, and 2.5 ml of 20% SDS, vortexed for 30 s and incubated at 65° C for 10 min. Supernatants from the two extraction cycles were pooled and extracted with an equal volume of chloroform-isoamyl alcohol (24:1 vol/vol). After extraction the aqueous phase was recovered and precipitated with 15 µg/ml of linear acrylamide and 0.6 volume of isopropanol. The resulting DNA pellet was resuspended in 0.5 ml TE [10 mM Tris (pH 8), 1 mM EDTA (pH 8)] and purified using columns supplied with the Ultra Clean Soil DNA Kit Mega Prep (MO BIO Laboratories, Inc., Solano Beach, CA), used according to the directions of the manufacturer.

DNA Extraction from LTCT tanks.
Genomic DNA was extracted from Long Term Corrosion Tanks (LTCT) using an extraction procedure developed to maximize the yield of microorganisms collected. DNA was extracted from 2 L of aseptically sampled solution removed from the tanks using a peristaltic pump and sterilized tubing. The sides of the tanks were also swiped with sterile gauze using a flamed-sterilized hemostat to remove microorganisms which may have colonized the tank walls. Gauze samples were stored in sterile Petri dishes prior to extraction. The solution sample (2 L) was vacuum-filtered through a sterile Durapore membrane filter (diameter 47 mm, pore size 0.2 µm; Milipore Corp., Bedford, MA). The filter was then placed in a 250 ml centrifuge bottle with the corresponding gauze and 50 ml of 0.1% sodium pyrophosphate [NaP_4O_7, (pH 7)] was added. The solution containing the filter and gauze was swirled vigorously for 15 min to desorb cells from the filter and gauze, which were subsequently removed. The resulting slurry was centrifuged at 6,000 X g for 10 min. to pellet cells. The resulting pellet was treated, DNA extracted and purified using the Ultra Clean Soil DNA Kit Mega Prep (MO BIO Laboratories, Inc., Solano Beach, CA), used according the directions of the manufacturer. Extracted and purified DNA was ethanol precipitated using linear acrylamide as a co-precipitant, and pellets were resuspended in a minimal volume TE before being subjected to Polymerase Chain Reaction.

Polymerase Chain Reaction (PCR) amplification of 16S rDNA
16S rRNA genes were amplified by PCR using a Eubacterial forward primer (B27f, 5'-AGAGTTGATCCTGGCTCAG-3', positions 8-27 *E. coli*) and a universal reverse primer (U1492r, 5'TACGGTACCTTGTTACGACTT-3', positions 1510-1491 *E. coli*)[8]. Each 50 µl reaction mixture contained 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM each deoxynucleoside triphosphate, 0.2µM of each primer, 2.5U of PLATINUM *Taq* polymerase (Life Technologies, Rockville, MD) and YM rock-extracted DNA template. PCR reaction conditions were: 5 min denaturation at 94° C., 30 s annealing at 65° C., 1.5 min extension at 72° C., followed by 28 cycles of 1 min at 94°C, 30 s annealing starting at 64° C. and decreasing at a rate of 0.4° C. per cycle to 55° C. at the 28th cycle. The final amplification cycle included 1 min denaturation at 94° C., 30 s. annealing at 55° C., with a final 10 min extension at 72° C.

Amplicon Cloning and Screening by Restriction Fragment Length Polymorphism (RFLP)
PCR products were purified by using QIAquick spin columns (Qiagen, Inc., Chatsworth, CA) according to the manufacturers instructions, and resuspended in sterile distilled water. Amplicons were cloned in pCR 2.1plasmid vector (Invitrogen, Inc., Carlsbad, CA), and the resulting ligation product was used to transform competent *E. coli* TOP10 cells. Recombinant plasmids were purified from over night cultures by using plasmid miniprep columns (Promega, Madison WI). YM rock or LTCT clones were subsequently purified and analyzed by RFLP by first amplifying cloned 16S rDNA inserts using M13 priming sites contained on pCR2.1[8], and then digesting the amplicons with *Rsa I. Rsa I* reaction products were analyzed by agarose gel (2.5%) electrophoresis. Clones showing unique RFLP patterns were then sequenced (MIDI Labs, Inc., Newark, DE).

Phylogenetic Analysis of Sequence Data

Each YM rock or LTCT clone sequence was submitted to the CHECK_CHIMERA program of the Ribosomal Database Project (RDP)[9] to detect the presence of possible chimeric artifacts. Questionable sequences were further investigated by examination of secondary structure and phylogenetic analyses of different portions of the sequences. Sequences were initially analyzed by using BLAST (National Center for Biotechnology Information) and SIMILARITY_RANK (RDP) searches to determine the closest available database sequences. For those sequences having a database relative showing a similarity value (S_{ab}) of >0.5, the highest scoring database sequence was obtained in aligned form from the RDP and placed into an alignment with YM rock or LTCT clone sequences. In addition, sequences representative of those major bacterial divisions not already included in the data set were obtained. Alignment of the final data set was accomplished by using Clustal W[10]. Sequence regions which could not be aligned with confidence, were excluded from the subsequent analyses. Similarity matrices were constructed from the aligned sequences by using only those positions for which there were data for 90% of the strains. Phylogenetic trees were constructed by the neighbor-joining method of Saitou and Nei[11].

RESULTS AND DISCUSSION

YM Bacterial Growth Rates in Simulated YM Groundwater

Previous experiments to assess the growth of microorganisms contained within YM rock under representative YM conditions utilized the growth medium "10XJ13 Synthetic." This medium was formulated to simulate *in situ* YM J13 well water increased 10-fold in concentration to mimic expected increases in groundwater ionic concentrations after nuclear waste emplacement (Table 1). Differences between the composition of analyzed J13 groundwater (increased in concentration 10X) and 10XJ13 Synthetic medium included decreased levels of sodium to preserve charge balance, decreased levels of calcium and carbonate to preclude precipitation of media components, exclusion of silica to control pH, and elimination of trace elements; 0.1% glucose was added to all media formulations (unless otherwise noted) to mimic organic carbon sources introduced into the repository (Table 1). These initial experiments established that there was enough nitrogen and sulfur in 10X groundwater to support significant microbial growth from YM rock. Exogenously added phosphate increased growth levels somewhat (1-2 orders of magnitude), but YM microbes were apparently able to solubilize enough phosphate from rock to increase bacterial densities in the aqueous phase to 10^8 cells/mL in batch experiments[12].

Bacterial growth rates from YM rock were then determined in unconcentrated simulated YM groundwater (1XJ13 Synthetic medium, a 1:10 dilution of 10X J13 Synthetic medium) with and without the addition of a carbon source under batch conditions. Exponential phase growth rates of planktonic YM organisms grown in 1XJ13 Synthetic medium either demonstrated doubling times of 2.3 hours (without glucose) and 1.7 hours (with glucose), with lag periods of one day (Fig. 1). Both cultures attained stationary phase in three to four days of incubation (Fig. 1). Stationary phase cell densities differed between cultures that contained glucose and those that did not by approximately one order of magnitude. Without glucose amendment maximal planktonic cell densities were on the order of from $4X10^6$ cells/mL to $8X10^6$ cells/mL, while with added glucose cell densities were about $2X10^7$ cells/mL to $7X10^7$ cells/mL (Fig. 1).

The addition of unconcentrated simulated groundwater thus resulted in significant growth from YM rock, even without an added source of organic carbon (Fig. 1). These results demonstrate that the primary limiting factor for microbial growth at YM is water availability. Prior experiments showed that of the major nutrients required for microbial growth (nitrogen, sulfur, phosphorous, and carbon), sufficient sources of both nitrogen and sulfur are available in a 10-fold concentration of YM groundwater, and phosphorous is apparently supplied by solubilization from YM rock[12]. Here, carbon-amended cultures, like phosphate-amended cultures[12], showed increases in final cell densities of 1-2 orders of magnitude. Therefore, in terms of their relative effects on the growth of YM organisms, water availability is the most

important factor, causing increases of at least 2-3 orders of magnitude beyond the estimated cell densities presently contained within rock (10^4-10^5 cells/gm dry rock[12]). Phosphate and organic carbon availability are secondary to the importance of water, each causing a 1-2 log increase in bacterial density over that attained without amendment of these nutrients.

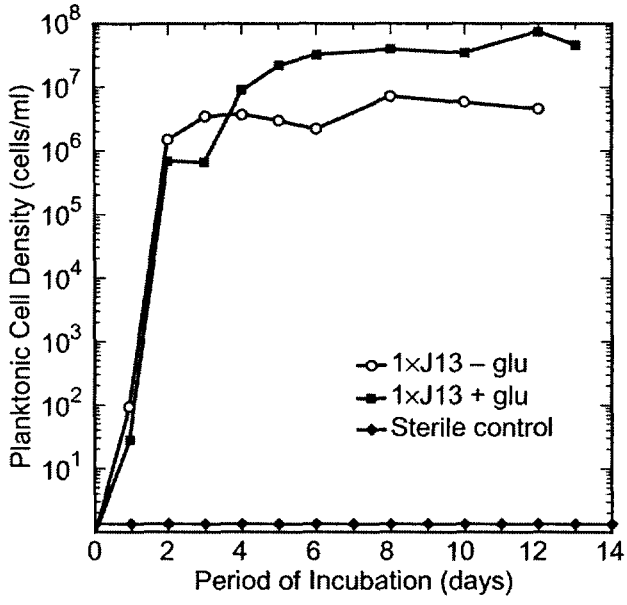

Figure. 1. Growth rates of YM aerobic bacteria in unconcentrated groundwater (1XJ13 Synthetic medium) with or without 0.1%glucose amendment, incubated under agitated batch conditions.
Sterile controls were prepared with pre-sterilized YM rock and incubated with 1XJ13 Synthetic medium with 0.1%glucose. Data shown was the average of two separate trials.

Bacterial Isolates Identified from YM Community Cultures

Stationary phase cultures grown in 1XJ13 Synthetic medium both with or without added glucose were subjected to single colony purification on R2A agar. An effort was made to single colony purify as many individual morphotypes as could be discerned, these were subjected to Biolog analysis to eliminate duplicate strains, and then identified using 16S rDNA sequence determination and analysis[6]. The strains that were thus isolated generally showed significant rDNA sequence divergence from database reference strains, demonstrating a 3.45% median divergence (3.88% average) from reference strains contained in the MicroSeq and Genbank databases (Table 2). While formally not considered identical species if sequence divergence is greater than 1%[13], for ease of reference these organisms are referred to here by their closest relatives.

Most of the organisms grown in 1XJ13 Synthetic with glucose differed from those isolated from 1XJ13 Synthetic without glucose, the single exception was a *Microbacterium keratanolyticum*-like bacteria. Even though identical organisms were not isolated from cultures containing glucose and those that lacked glucose, most (three out of five) of the isolates cultured from 1XJ13 Synthetic without glucose correlated to phylogenetically-related isolates from 1XJ13 Synthetic with glucose (Table 2).

While the effort to isolate and identify organisms growing from YM rock in simulated ground waters was constrained by selection on a single media type under aerobic conditions, the types of organisms that were found and identified, and the comparisons among them may be significant to repository performance. All of the organisms that were isolated appear to be commonly found in soils,

and are adapted to low nutrient conditions. Many of the isolates have the capabilities to degrade complex organic compounds, including ethers, aromatic compounds, and halogenated chemical species[14]; physiological diversity has also been observed among organisms isolated from other subsurface sites[15]. *R. eutrophus* (formerly known as *Alcaligenes eutrophus*) includes some strains that are resistant to heavy metals[16].

Table 2. Organisms isolated after growth in various YM simulated groundwaters and 16S rDNA sequence divergence from reference organisms

Organism[a]	Growth Medium from Which Organism Isolated	
	1×J13 Synthetic with glucose	1×J13 Synthetic without glucose
	% divergence from data base[b]	
Ralstonia pickettii		
Ralstonia eutrophus	4.5/MS	6.9/MS
Burkholderia cepacia		
Blastobacter natatorius		
Sphingomonas paucimobilis		2.0/GB
Methylobacterium mesophilicum		
Caulobacter subvibroidies		
Uncultured bacterium oxSCC–6[c]	4.0/GB	
Pseudomonas (Janth) *mephitica*	6.06/MS	
Microbacterium barkeri	4.55/MS	
Microbacterium keratanolyticum	4.15/MS	4.15/MS
Microbacterium chocolatum	5.16/MS	
Arthrobacter sp. SMCC G964[d]	0.0/GB	
Pseudomonas stutzeri	3.93/MS	1.0/GB
Afipia genosp. 14		4.0/GB

[a] Closest relative in 16S rDNA sequence comparisons to three separate databases (i.e., MS, GB, RDP, below)

[b] MS, MicroSeq database; GB, GenBank database; RDP, Ribosomal Database Project

[c] Lüdemann, H., I. Arth, and W. Liesack. 2000. Spatial changes in the bacterial community structure along a vertical oxygen gradient in flooded paddy soil cores. Appl. Environ. Microbiol. 66: 754-762

[d] VanWaasbergen, L. G., D. L. Balkwill, F. H. Crocker, B. N. Bjornstad, and R. V. Miller. 2000. Genetic diversity among *Arthrobacter* species collected across a heterogeneous series of terrestrial deep-subsurface sediments as determined on the basis of 16S rRNA and *recA* sequences. Appl. Environ. Microbiol. 66:3454-3463.

Ribosomal DNA (rDNA) Analysis of YM Bacterial Communities

It has been commonly acknowledged that only a small fraction of organisms extant in any given environmental sample grow and can be isolated on a single type of growth medium[2]. Therefore, methods that utilize direct DNA extraction from environmental samples, with subsequent

characterization of the 16S rDNA genes contained within the obtained DNA, have been developed to better assess community compositions without the need to grow and isolate organisms[17]. Characterization of DNA extracted directly from YM rock was therefore undertaken to generate a more complete roster of the organisms contained within the YM community, and thereby provide more accurate knowledge of the classes of organisms extant within the prospective repository. Metabolic activities associated with identified organisms will guide a better understanding of their potential impacts on nuclear waste packaging materials.

Four hundred grams of YM rock was subjected to extraction, the resulting DNA was purified and subjected to a series of biochemical and genetic manipulations that included amplification of bacterial 16S rDNA genes and cloning amplified genes. Two hundred rDNA clones were screened to that obtain those that were unique based on Restriction Fragment Length Polymorphisms (RFLP). Statistical analysis confirmed that, having screened 200 clones, those organisms comprising greater than 3.5% of the community have a 0.1% probability of not being detected. Conversely, those organisms that comprise less than 0.5% of the YM community have a 37% of not being detected. Therefore, while screening 200 clones may not detect *all* organisms extant at the site, the vast majority of organisms were detected, and only those that represent a very small percentage of the total microbial community would not have been detected and identified.

After screening 200 rDNA clones, 65 unique clonal types, representing 65 separate types of organisms, were distinguished. The frequency of occurrence during screening may indicate the relative abundance of these organisms in the YM rock samples (Fig. 2). Two clonal variants (*Arthrobacter nicotianae* and *Microbacterium imperiale*) accounted for almost 33% of the total number clones screened, four other clones (*Devosia riboflavin, Bacillus aminovorans, Eschericia coli*, and *Ralstonia taiwanensis*) occurred at moderate frequency (3-10% of total number of clones screened). 54% of the screened clones occurred at low frequency (<5 occurrences). If biases in DNA extraction and amplification are ignored, the frequency of their occurrence would directly reflect their relative abundance in the YM environment; thus *A. nicotianae, M. imperiale*, and *D. riboflavin* could be assumed to be the most abundant organisms in the examined community.

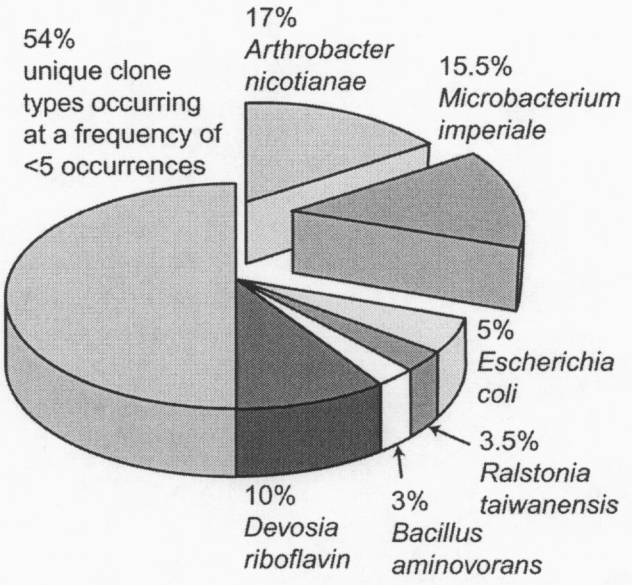

Figure. 2. Frequency of unique clone types in YM rock (of 200 screened). After amplification and cloning of 16S rDNA genes, 200 clones were screened; the frequency of occurrence of unique clone types is depicted as the percentage of the total number of clones screened.

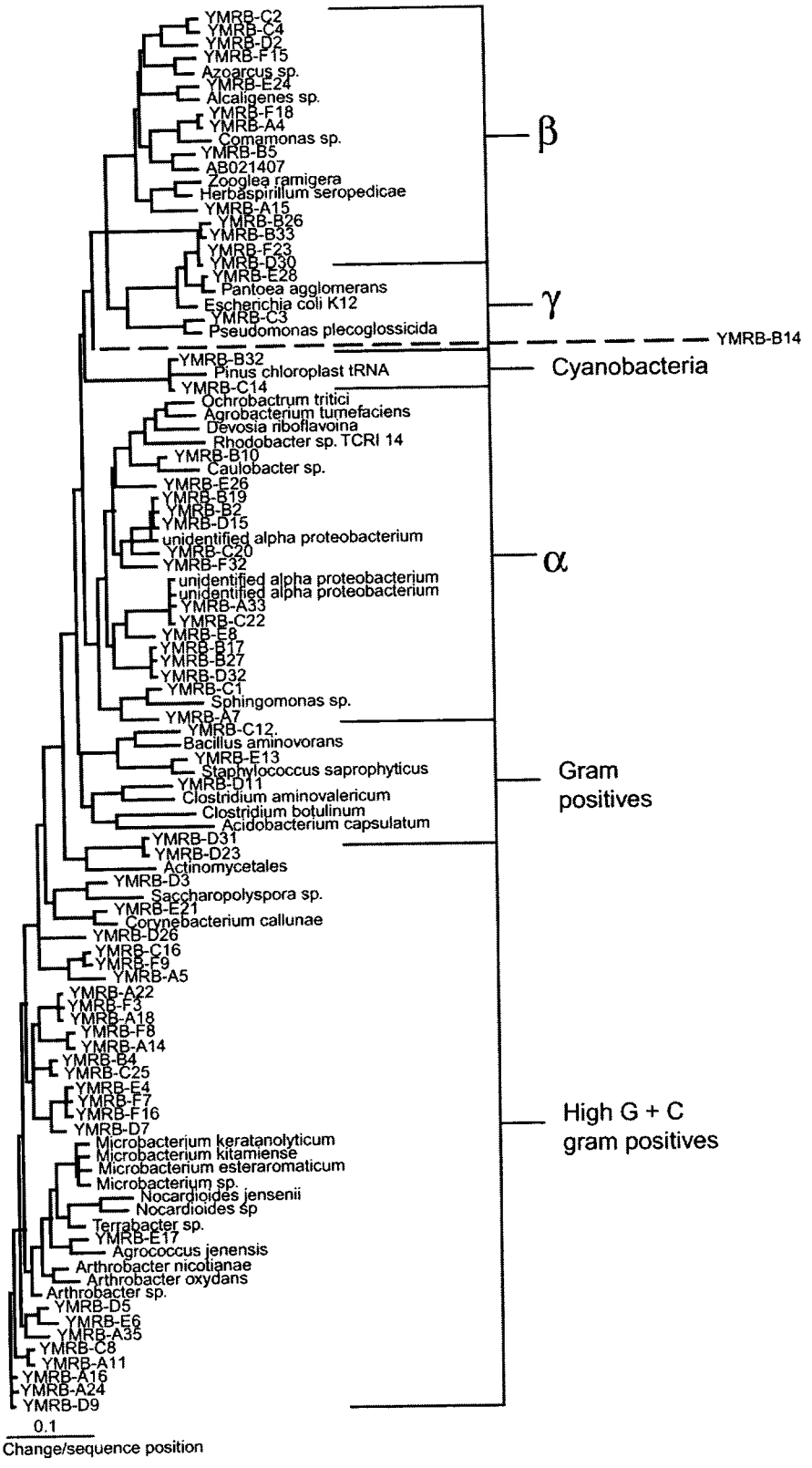

Figure. 3. Phylogenetic tree of YM bacterial community as identified by 16S rDNA analysis of DNA extracted from YM rock. Named species are those extant in the RDP database[9] to serve as reference benchmarks, while "YMPRB-" designated strains were those identified from YM rock. Lengths of horizontal branches denote relative time since divergence from common ancestors (nodes). Large phylogenetic groupings are provided on the right-most margin.

After sequencing, an evolutionary (or, phylogenetic) tree of all unique cloned sequences was generated, together with related reference organisms from the rDNA database[9] (Fig. 3). These trees show the relatedness between the organisms that were identified and known extant organisms; nodes denote common evolutionary ancestors while the length of branches connote evolutionary time since divergence from a common ancestor. Identified YM organisms span a wide phylogenetic range, and include groups of organisms known to reside in dry environments (e.g., the High G+C Gram Positives), as well as those that sporulate into resistant forms that can survive desiccation and elevated temperatures (e.g., the Bacilli and Clostridia). When collecting rock samples for extraction, no effort was made to isolate purely endogenous YM organisms; both those from tunnel walls (which may be introduced during construction) and those within the rock were collected. Clearly, at least two identified YM organisms were introduced into the repository, these were related to photosynthetic cyanobacteria which may be able to survive due to the artificial light or could be plant pollens that were introduced by ventilation of the ESF. In any case, the metabolic pathways inherent to identified organisms are currently being explored to assess their potential effects on waste package materials.

Ribosomal DNA (rDNA) Analysis of Microbial Communities in Long term Corrosion Test Environments

Long term corrosion tests (LTCT) of candidate waste package materials are being conducted in laboratory environments that are intended to simulate expected repository conditions. While these test environments (contained in 1000 L tanks) did not intentionally incorporate microorganisms, preliminary microscopic analysis showed that organisms were present in at least some 60° C. tank environments (data not shown). Therefore, as an indication of what organisms might re-colonize the repository after the initial radiation and heat pulse have dissipated, test tank environments are being analyzed for their full microbiological complement using 16S rDNA analysis.

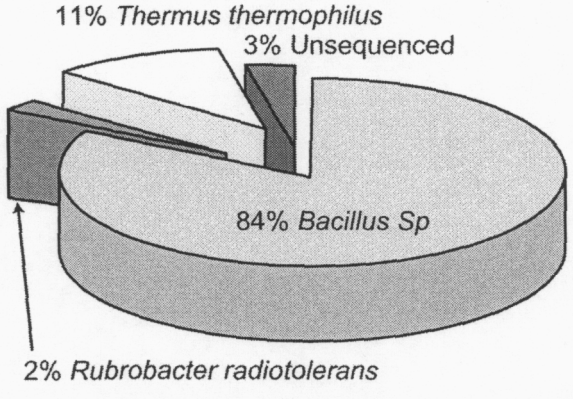

Figure 4. Frequency of unique clone types in a LTCT tank containing simulated 10X groundwater at 60° C. (of 100 screened). After amplification and cloning of 16S rDNA genes, 100 clones were screened; the frequency of occurrence of unique clone types is depicted as the percentage of the total number of clones screened.

After screening 100 clones obtained from a LTCT being conducted using a test solution which is close in composition to that of 10X J13 Synthetic medium and maintained at 60° C., five different bacterial inhabitants were discerned (Fig. 4). If the frequency of occurrence during the screening process is taken as an indicator of relative abundance of the respective organisms in the tank environment (see above), then a group of closely related Bacilli were observed to be most numerous, comprising 84% of the clones screened (Fig. 4). Bacilli, as mentioned previously, are able to form heat- and resistant-resistant spores which enable them to survive through adverse environmental conditions, they have also been associated with high temperature environments, such a hot springs[18]. An extremely heat-tolerant organism closely related to *Thermus thermophilus*, comprised 11% of the clones surveyed. *T. thermophilus* grows at temperatures ranging from 50° to 85° C., with a temperature

optimum of 70° C.[19] The presence of these organisms demonstrates that if organisms are introduced to environments where conditions are selective for their survival, that those organisms best adapted to prevailing conditions will be successful in becoming established. An organism which is known to be both heat- and radiation-resistant, *Rubrobacter radiotolerans*[20], was also found to reside in this test environment.

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